## Sniffing out contaminants

***Spiros Paramithiotis, Natasha Spadafora, Carsten Muller, Eleftherios H. Drosinos*** *and* ***Hilary Rogers*** *look at current methods and future prospects for detecting Listeria in fresh fruit and vegetables.*

A key component of the EU and WHO strategy to promote a healthy diet includes sufficient intake of fresh fruit and vegetables[1] with a daily recommended dose of 400 g. Recently, the consumption of ready to eat salads and fruit salads has been rising in Europe, the USA and elsewhere. These products help to provide readily accessible sources of fruit and vegetables in the home and on the move. However, both traditional fresh fruit and vegetables and minimally processed products can harbour human pathogens. Minimally processed foods are particularly vulnerable compared to fully processed foods, as they do not typically include a kill step to eliminate the presence of micro-organisms, as this would also affect their nutritional value. Furthermore, due to the processing steps that include trimming, peeling and cutting and then a wide distribution of the product, opportunities for propagating any contaminants entering the supply chain are increased[2]. A key priority for the industry therefore is early detection of contaminants in the supply chain and a thorough investigation of any contamination found to determine the source. Processes can then be improved to reduce occurrence of contamination to a minimum.

### Pathogens associated with fresh fruit and vegetables

The principal pathogens of concern in the safety of fresh fruit and vegetables are *Escherichia coli,* especially the most virulent strains such as O157, *Salmonella* serovars, and *Listeria monocytogenes*[3]. Listeria presents particular problems because it is able to grow even at the low temperatures used in the supply chain for minimally processed foods[4]. *L. monocytogenes* is the etiological agent of foodborne listeriosis, which annually results in hundreds of illnesses, hospitalisations and deaths worldwide. Prevalence in fresh fruits and vegetables has been reported to vary from 0.04% to 36.8%[5] depending upon a variety of factors including agricultural practices and logistics conditions[6].

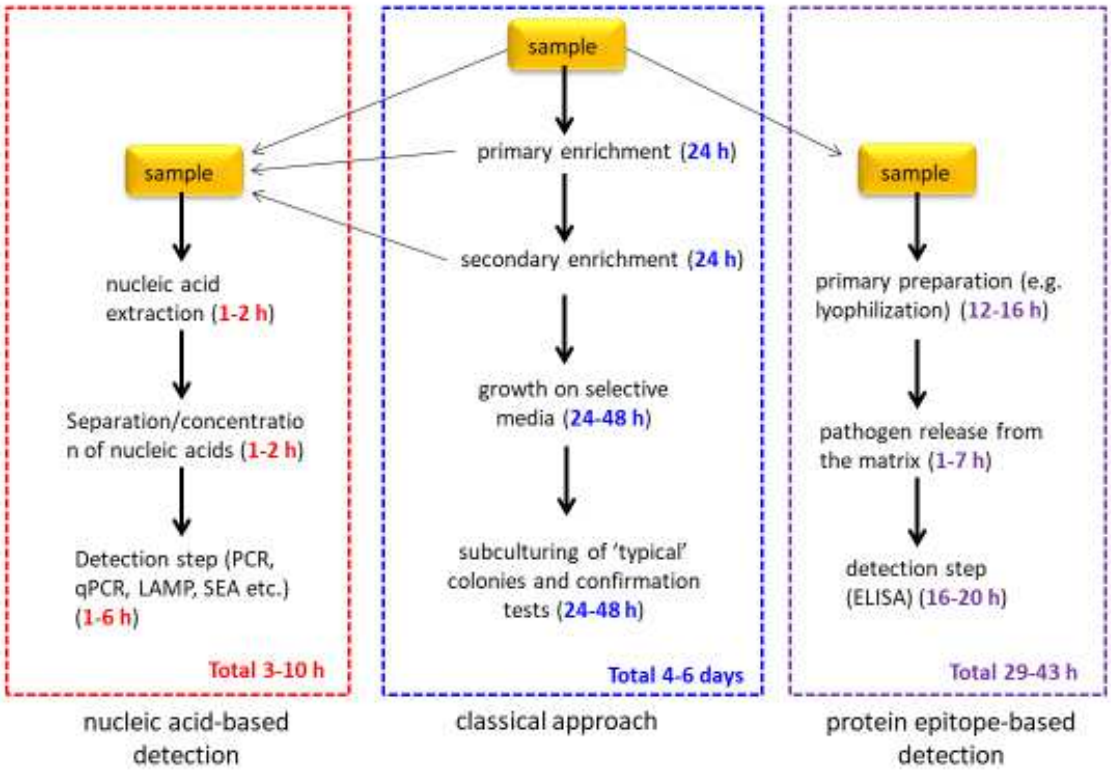
Over the last decade several outbreaks of listeriosis have been reported, the most pronounced of which were in the USA (the multi-state 2011 cantaloupe and lettuce outbreaks), the 2017-18 South African outbreak (polony processed meat sausage) and in Europe the multi-country 2018 frozen corn outbreak. In the first case, a total of 231 hospitalisations and 48 deaths were reported[7] while in the South African outbreak, 216 deaths, including at least 92 children, have been recorded to date[8]. The European outbreak resulted in 47 hospitalisations and 9 deaths[9]. These highlight the importance of fast and reliable detection of the pathogen.

**Detection of *Listeria***

Presence of the pathogen and its population size depends upon the hygienic conditions during production, processing and distribution as well as the intrinsic characteristics of the product (pH, water activity, presence of antimicrobial substances etc.). In the majority of cases, the population of the bacterium may not exceed a few cells per 25g of product. In addition, another dominant microbial population may be present at numbers several orders of magnitude higher. Furthermore, it is very likely that some or all of the cells of the pathogen are injured or may have entered a viable but non culturable state because of the aforementioned intrinsic properties of the commodity or its processing.

Strategies that have been employed for detection of *Listeria monocytogenes* are summarised in Figure 1. The classical approach aims to detect the pathogen itself. For that purpose, two enrichment steps are necessary to allow the pathogen to regain viability and/or culturability and increase in numbers while at the same time suppressing the rest of the microbial populations. Then, presence is assessed through cultivation on selective solid media and confirmation tests on colonies exhibiting a typical appearance according to the medium used[10]. This is the reference method and no additional skills are required for its execution and interpretation apart from a microbiological background. On the other hand, a conclusive result may be reached only after 6-7 days. This is a time-frame that exceeds the shelf-life of many fresh cut fruits and vegetables and is a particular problem for the fresh cut industry where products may only transit at the processing site for 1-2 days.

**Figure 1. Overview of different strategies for the detection of *Listeria monocytogenes* in fresh fruit and vegetables with estimated times for each step.**

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## Rapid detection methods

To reduce the time frame, development of alternative strategies that may allow faster detection of listeria has been extensively researched. Indeed, a wide variety of methods have been proposed based either on the detection of nucleic acid sequences, mostly DNA, or protein epitopes usually residing on the cell surface, both of which need to be specific for the pathogen. In the first case, many protocols based on quantitative and qualitative polymerase chain reaction (PCR) formats, loop-mediated isothermal amplification (LAMP), fluorescence *in situ* hybridisation (FISH) and

strand exchange amplification (SEA) have been developed[11],[12]. Similarly, the detection of protein epitopes through a wide variety of ELISA protocols has been reported[13].

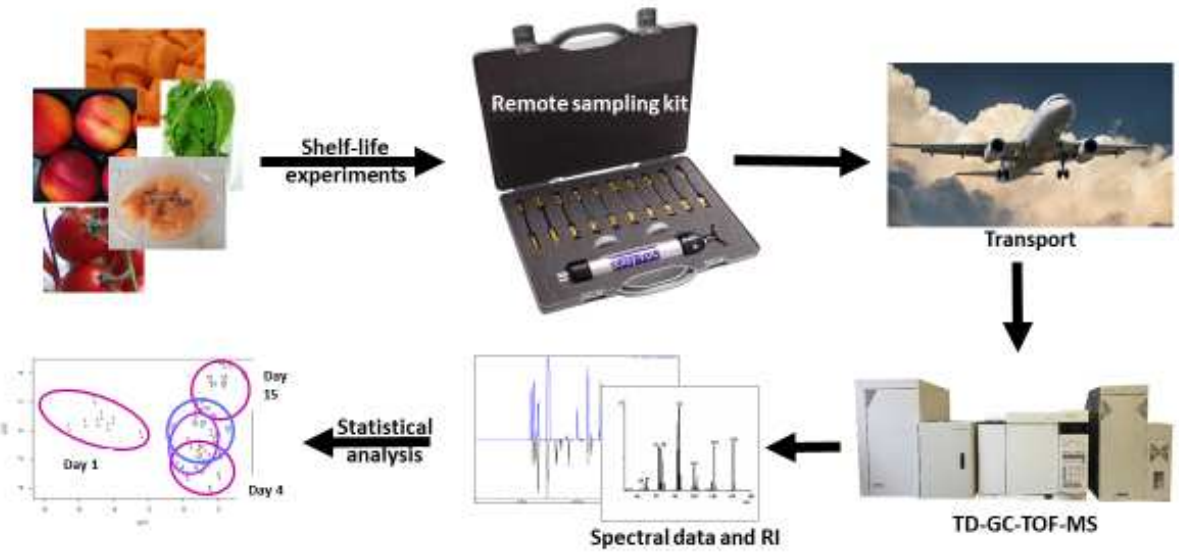
For all the DNA or protein based assays, the detection protocol itself requires less than a day; however selective enrichment is also necessary for the reasons already mentioned. Alternatively and/or additionally, separation and/or concentration of the target cells may be necessary in order to remove interfering molecules and concomitantly improve target detectability as well as method sensitivity and specificity. However, the addition of such steps increases the time required for the analysis, as well as the complexity and the level of expertise required. At the same time, the sensitivity and specificity of these methods are inferior to the reference method, mostly due to primer specificity in nucleic acid detection and cross reactivity between the antibodies and other plant material in protein detection.

**Measuring VOCs to detect *Listeria***

Analysis of volatile organic compounds (VOCs) provides an alternative option for detection of *Listeria,* which would not require selective enrichment. An advantage of VOCs is that they can be collected in a non-destructive way directly from the product and their analysis is rapid (Figure 2). Thus in principle, batches of product could be analysed routinely for changes in VOCs indicative of contamination with human pathogens. The profile of volatile organic compounds (VOC) alters during the shelf-life of fresh cut products[14],[15] at least in part due to the growth of spoilage microflora. This can be detected for example as off–odours late in shelf life of bagged salads. However, using objective instrument-based assessment of the VOCs, very subtle changes in profile can be detected and used to assess quality of the product. Although bacteria such as *L. monocytogenes* are not considered spoilage bacteria, they can multiply on the surface of fruit and vegetables, producing a range of VOCs including alcohols, amines esters, hydrocarbons, and ketones[16]. Detection of bacteria other than *Listeria* using VOCs has been reported in a range of products including mango fruit, tinned tomatoes and strawberry. In most of this work, detection used gas chromatography mass spectrometry (GC-MS) using solid phase micro extraction (SPME) or electronic noses. In some cases, specific compounds could be associated with the specific microorganism, however these associations are often not completely robust when analysed across

different matrices or conditions. Even subtle changes in the relative abundance of a common set of VOCs could be used to detect the presence of human pathogens and indeed may be more robust and specific[17].

**Figure 2. VOC collection and analysis for assessing quality and safety of fresh fruit and vegetables. VOC samples are collected onto SafeLok thermal desorption (TD) tubes packed with Tenax TA and SulfiCarb sorbents using an EasyVOC™ pump (Markes International Ltd). Typically 200ml to 1L of headspace are collected. Tubes can be safely transported to the laboratory where VOCs are desorbed using thermal desorption and analysed by gas chromatography (GC) to provide spectral data. Time of flight mass spectrometry (TOF-MS) is then used to identify the VOCs based on comparison to databases such as NIST. Statistical analysis using R software and based on PerMANOVA and CAP allows discrimination amongst different samples.**

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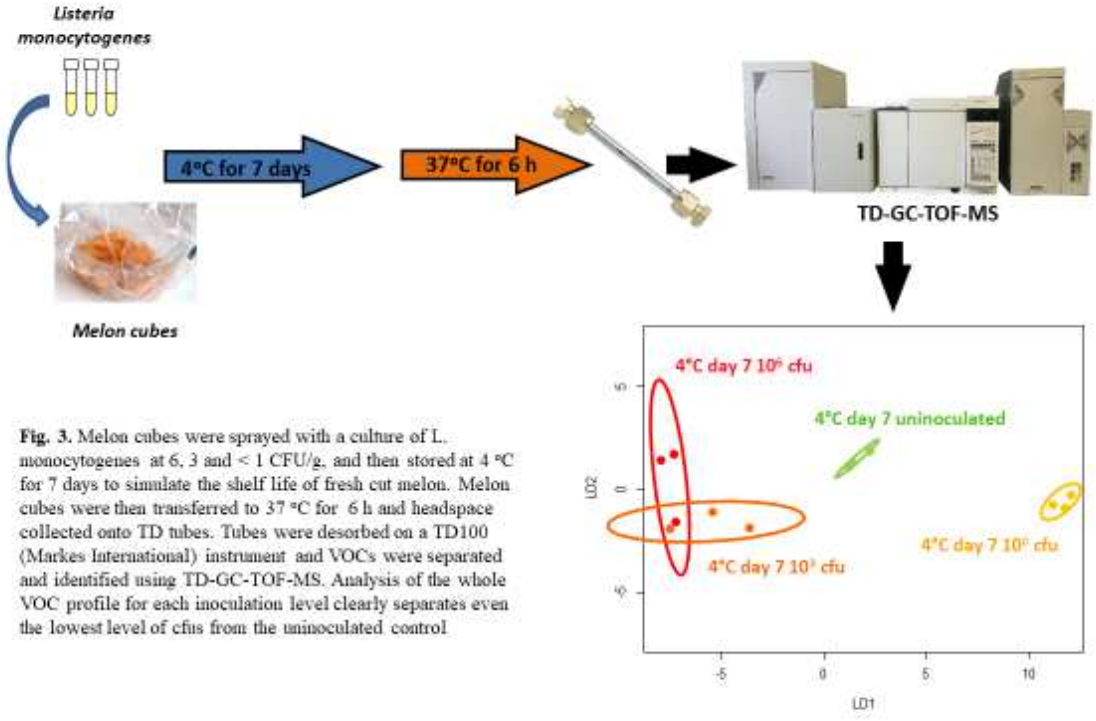
Detection of *L. monocytogenes* using VOCs on cantaloupe melon at levels lower than 1 log CFU/g was reported recently by the current authors[18] (Figure 3). The study used a combination of VOC collection on thermal desorption tubes with analysis on GC-MS and a time-of-flight MS (TD-GC-TOF-MS). This approach provides a platform that combines quick, simple and stable sampling of VOCs on site with a much more sensitive detection than other forms of mass spectrometry. In addition, the detection of *Listeria* was based initially on differences detected between complete VOC profiles using multivariate statistical methods, such as PerMANOVA and CAP for analysis. Further statistical evaluation of profiles (WCNA) enabled identification of a panel of VOCs that discriminated between contaminated and uncontaminated melon samples. The methodology required 6h incubation for enrichment of VOCs and 1h analysis time, much faster than current culture-based approaches. This time-frame falls within the required needs of the processing industry and is comparable to DNA and protein-based methods discussed above.

**Figure 3. Melon cubes were sprayed with a culture of *L. monocytogenes* at 6, 3 and < 1 CFU/g, and then stored at 4 ºC for 7 days to simulate the shelf life of fresh cut melon. Melon cubes were then transferred to 37 ºC for 6 h and headspace collected onto TD tubes. Tubes were desorbed on a TD100 (Markes International) instrument and VOCs were separated and identified using TD-GC-TOF-MS. Analysis of the whole VOC profile for each inoculation level clearly separates even the lowest level of cfus from the uninoculated control.**

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One of the most difficult problems for the industry is false-negative results. Contaminants can be missed due to the difficulty of sampling product batches exhaustively when the microorganisms are present at very low levels. VOC analysis may be able to help with this by enabling detection from larger pooled product batches although further research is needed to verify the robustness of the technique at the industry scale. Furthermore, the method could be used to calibrate other detection methods e.g. PTR-MS, SWIFT-MS, FAIM or eNoses. These can all be used onsite without the need to transport samples and offer much cheaper alternatives to the investment in a TD-GC-TOF-MS system. Moreover, they could be calibrated to detect changes in overall quality of the product as well as the presence of microorganisms thus adding value for the industry and the consumer.

Thus, VOC analysis could be a useful new approach to add to the tool box for contaminant detection, complementing existing analytical techniques. It can provide valuable independent extra confirmation of results based on other rapid detection methods to help to ensure that consumption

of fresh fruit and vegetables is safe. This will ensure that we can continue to eat our daily 400 g of fresh fruit and vegetables, known to protect against a range of important long-term conditions, such as cancer, diabetes, and cardiovascular disease, without the fear of exposure to food-borne pathogens.

**Spiros Paramithiotis[1], Natasha Spadafora[2,3], Carsten Muller[2], Eleftherios H. Drosinos[1], Hilary Rogers[2]**

[1]Laboratory of Food Quality Control and Hygiene, Department of Food Science and Human Nutrition, Agricultural University of Athens, Iera Odos 75, GR-118 55 Athens, Greece

[2]School of Biosciences, Cardiff University, Sir Martin Evans Building, Museum Avenue, Cardiff CF10 3AX

[3]Markes International Ltd, Gwaun Elai Medi-Science Campus, Llantrisant, RCT, CF72 8XL

**Lead Author's area of expertise:** Hilary Rogers is a plant biologist interested in understanding the molecular and biochemical changes occurring in fresh produce during post-harvest senescence.

**Email:** rogershj@cardiff.ac.uk; **Tel:** +44 (0)2920876352;

**Web:** http://www.cardiff.ac.uk/people/view/81265-rogers-hilary

**Key words:** *Listera monocytogenes*, fresh cut salads, volatile organic compounds, food-borne pathogens

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